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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have investigated the potential of genistein, the primary phytoestrogen component of soy, to protect against chemically-induced prostate cancer in rats. Lobund-Wistar rats were exposed to 0, 25 and 250 mg genistein/kg AIN-76A diet, starting at conception and continued until necropsy at 11 months. Male offspring were injected s.c. with Flutamide on days 50-66 to effect chemical castration, with testosterone on days 67-69 to stimulate cell proliferation, with N-methylnitrosurea (NMU) into the dorsolateral prostate to initiate cancer causation, and given testosterone implants, starting at day 77 to promote the cancer. The percent of tumors to the prostate that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250/kg mg genistein/kg diet were 77.3%, 61.1%, 44.4%, respectively. Genistein did not alter body, prostate or testes weights or feed consumption. Male rats fed 0, 25 and 250 mg genistein/kg diet had serum genistein concentrations of 9, 60 and 861 pmol/ml, and prostate genistein concentrations of 85, 230 and 775 pmol/g tissue. We conclude that lifetime exposure to "physiologic" concentrations of genistein in the diet protected against chemically-induced prostate cancer development in rats without significant toxicity to the offspring.

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INTRODUCTION

Asian men consuming a diet high in soy products have a lower incidence of clinically manifested prostate cancer compared to American and European men (1-3). Soy based diets are high in phytochemicals and quantitative results indicate that genistein is the primary isoflavone constituent of human urine from subjects consuming large amounts of soy products (tofu, soy flour, soy milk, tempeh, etc) (4,5). While the disease of prostate cancer is usually attacked at time of diagnosis, we look at this existing problem from a new perspective. We believe that predisposition to cancer (and its prevention) occurs early in life. We have hypothesized that exposure of male rats to genistein, starting *in utero,* will confer protection against prostate cancer. The aims of this study were: 1) to investigate the potential of life-time exposure to genistein in the diet to reduce susceptibility for prostate cancer, 2) to determine if genistein would regulate the EGFR-signaling pathway as the mechanism of action, and 3) to measure the concentrations of genistein and its metabolites in blood and dorsolateral prostate of male offspring.

BODY

Genistein Exposure. Lobund-Wistar rats were provided 0, 25 and 250 mg genistein/kg AIN-76A diet, starting at conception. Exposure to genistein in the diet, *in utero* until time of carcinogen administration at 70 days postconception, did not cause significant alterations to body weights (Fig. 1). Likewise, perinatal exposure to genistein in the diet did not cause significant alterations to the testes and prostate weights in 70 day old rats (Table 1). The daily feed consumption was measured in young male rats. All animals ate approximately the same amount of feed and gained the same amount of weight (Table 2). From this data we calculated that young male rats fed the low and high genistein containing diets ingested 1.80 mg and 19.25 mg genistein/day/kg BW, respectively.

Prostate Cancer Protocol. From day 50-66 postpartum, male offspring were gavaged with 33 mg Flutamide/kg BW to effect chemical castration. On days 67, 68, and 69, they were injected with 25 mg testosterone/kg BW to stimulate cell proliferation. On day 70, all rats were anesthetized and 42 mg N-methlynitrosourea (MNU)/kg BW was injected into the dorsal prostate for cancer initiation. One week after MNU administration, silastic implants of 25 mg of testosterone were implanted (and replaced every 12 weeks) to stimulate mitosis and promote tumor growth. Animals were necropsied when 48 weeks old or when animals became moribund. Continuation of genistein in the diet to adult rats on the carcinogenesis protocol did not significantly alter body, testes and dorsolateral weights compared to rats fed AIN-76A diet only (Table 3).

Tumor Induction. Except for two animals, one with ganglioneuroma and the other with malignant peripheral nerve sheath tumor, prostatic carcinoma was the sole malignancy identified in these animals. Male offspring exposed from time of conception until 11 months old to genistein in the diet and treated with the carcinogenesis protocol had lower incidence of prostate tumors than those fed the control diet and treated with the same carcinogenesis protocol (Table 4). Rats exposed to 0, 25 and 250 mg genistein/kg diet had tumor incidence of 86.4%, 77.8%, and 63.0%,

respectively. Prostate tumor weights from the 3 groups were not significantly different from one another (5.2 ± 1.8 grams). In animals with small tumors, the tumors were confined to the site of NMU injection, the dorsolateral prostate.

The percent of tumors to the prostate that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250 mg genistein/kg diet were 77.3%, 61.1%, and 44.4%, and the incidence of intraductal carcinomas were 55.5%, 52.9% and 34.5%, respectively (Table 4). Comparison of tumor invasive adenocarcinomas from rats exposed to 250 mg genistein/kg diet to no genistein in the diet was statistically significant to 0.04. In the majority of animals, the tumors presented as Stage I (55%) (confined to the dorsolateral prostate), and in (33%) as stage II and in few (12%) as stage III. Genistein had no effect on the stage of the tumors. Unlike human prostatic carcinomas, which may show all grades, prostatic tumors in these animals presented mostly as poorly differentiated carcinomas. They were composed of small irregular glands and cluster of atypical cells infiltrating a dense stroma. There was extensive acute inflammation, necrosis and calcification. Mucous secretions as well as signet cells were occasionally present. The histology did not vary between animals, or between stages. The lowest score we observed was 6 and the majority between 8 and 10. We found no difference in Gleason's score of tumors in genistein-treated and -untreated animals. Mild to moderate dysplasia was seen less frequently in treated (63%) than untreated (81%) animals.

The prostate tumors did metastasize to the other organs of the reproductive tract, i.e., ventral prostate, seminal vesicles, vas deferens, bulbourethral gland, coagulating gland, epididymis, testes, and sometimes to the penis. There was also metastasis to the lymph nodes, bladder, adrenal gland, diaphragm, kidney, liver, lungs, mesentery, and spleen. There was no evidence of metastasis to the bone or brain. These histopathological findings are consistent with those of Schleicher et al (6).

EGF-receptor expression. Western blot analysis for the EGF-receptor in dorsolateral prostates from rats fed 0, 25 and 25 mg genistein/kg diet did not show any significant differences between the groups.

Bioavailability. Dietary genistein concentrations of 25 and 250 mg genistein/kg diet resulted in serum total genistein concentrations of 60 and 861 pmoles/ml (Table 5). These "frame" the human circulating genistein concentration of Asian men (276 pmol/ml) eating a diet high in soy (7). Enzymatic hydrolysis of the serum with beta-glucuronidase and sulfatase, and HPLC-mass spectrometry analysis (8) revealed that only 15% and 8% of the genistein was free- or aglycone-genistein from blood of rats fed 25 mg and 250 mg genistein/kg diet, respectively, indicating that most of the circulating genistein is conjugated.

In the prostate of rats fed the high genistein containing diet (250 mg/kg diet), genistein concentrations were measured to be 775 pmole/g tissue. This is similar to the corresponding blood levels, 861 pmoles/ml. This shows that there is no genistein transport barrier to the prostate. Another significant finding is that all of the genistein in the prostate was in the free

form (774 pmol/g), none was conjugated. This suggests that all of the genistein is available to the prostate for direct biological action.

Experiments into sex steroid dependent and independent signaling pathways, and down-stream events are necessary. Likewise, investigations into genistein timing of exposure for chemoprevention (prenatal, neonatal, prepubertal and adult) are also warranted.

KEY RESEARCH ACCOMPLISHMENTS

1) A rodent model was developed for the study of prostate cancer. Intraprostatic injection of NMU in rats resulted in 77% incidence of invasive adenocarcinomas originating in the dorsolateral prostate.

2) Life time exposure to dietary genistein significantly suppressed the development of chemically-induced prostate tumors (invasive adenocarcinomas).

3) The concentrations of genistein to exert this chemoprevention resulted in circulating genistein concentrations similar to those found in Asian men eating a diet containing high concentrations of soy. Genistein was bioavailable to the prostate.

4) Perinatal exposure to genistein in the diet did not cause significant toxicity to the offspring.

REPORTABLE OUTCOMES

1) Abstract: Wang, J., Eltoum, I.-E. and Lamartiniere, C. A. Dietary genistein suppresses chemically-induced prostate cancer development in Lobund-Wistar rats. Proceedings of the American Association for Cancer Research. 42:461, 2001.

2) One manuscript submitted to Cancer Letters: Dietary genistein suppresses chemically-induced prostate cancer in Lobund-Wistar rats. Authors: Wang, J., Eltoum, I.-E. and Lamartiniere, C. A.

3) Also, this data have been presented at the following meetings:

University of Missouri Sixth Annual Oncology Conference. Dietary Genistein Protects against Mammary and Prostate Cancers. Lake of the Ozarks, Missouri. April 27, 2001

FASEB Summer Research Conference on Physiological Functions of Antioxidant Nutrients and Phytochemicals. Genistein and Breast and Prostate Cancers. Tucson, AR. June 16-21, 2001

Hormonal Carcinogenesis Gordon Conference. Dietary Factors in Hormonal Carcinogenesis. Genistein Chemoprevention: Timing of Exposure and Mechanisms of Action (Mammary and Prostate). Kimball, NH, July 8-13, 2001.

PERSONNEL SUPPORTED

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CONCLUSIONS

The study of cancer of the prostate is hindered by a lack of adequate animal models. Using intraprostatic injection of the carcinogen, NMU, we have developed a rodent model that resulted in 77% incidence of invasive adenocarcinomas originating in the dorsolateral prostate. Then, we demonstrated that life time exposure to dietary genistein significantly suppressed the development of chemically-induced prostate cancer in rats. Measurements of serum and prostate genistein concentrations showed circulating genistein concentrations similar to those found in Asian men eating a diet containing high concentrations of soy (7), and that genistein was bioavailable to the prostate. Perinatal exposure to genistein in the diet did not cause significant toxicity to the offspring. This data supports the epidemiological reports that soy (1-3), or genistein, protects against prostate cancer.

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APPENDICES

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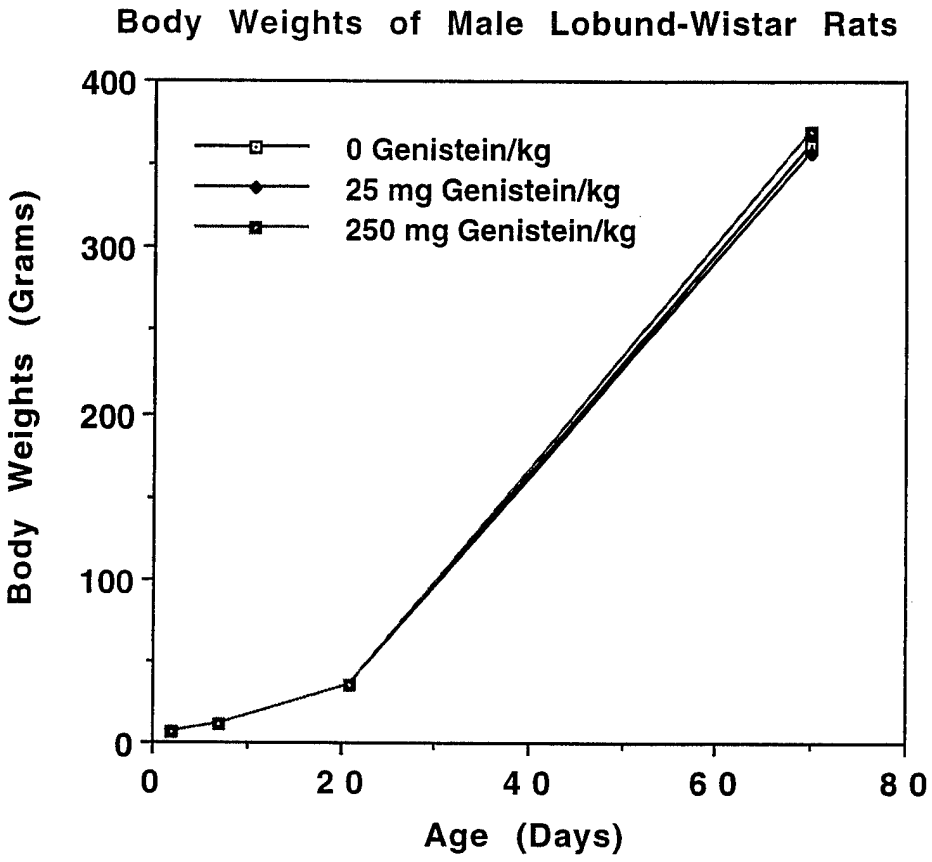

Figure. 1. Body weights of male Lobund-Wistar rats exposed to genistein in the diet from conception until 70 days old. The dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 postpartum.

Table 1. Body, Testes and Dorsolateral Prostate Weights in Lobund-Wistar Rats Exposed to Genistein in the Diet from Conception until 70 Days Old*

Diet	Body Weights (G)	Testes (G)	Dorsolateral Prostate (mg)
Zero Genistein in AIN-76A	244 ± 8	2.30 ± 0.04	190 ± 4
25 mg Genistein/kg AIN-76A	256 ± 4	2.35 ± 0.04	188 ± 5
250 mg Genistein/kg AIN-76A	254 ± 3	2.33 ± 0.02	186 ± 5

*Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum.

Table 2. Dietary Genistein Consumption in Lobund-Wistar Male Rats*

Diet	Average BW over Period	Grams of Diet Eaten/Day/Rat	Grams of Diet /Day/kg BW	Grams of Genistein Eaten/Day/kg BW
Zero mg genistein/kg diet	232 ± 6	17.9 ± 0.7	77	0
25 mg genistein/kg diet	235 ± 6	17.0 ± 0.6	72	1.80
250 mg genistein/kg diet	231 ± 6	17.7 ± 0.8	77	19.25

*Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum. Feed consumptin was measured from 60-70 days postpartum. Each value represents 8 rats/group.

Table 3. Body, Testes and Dorsolateral Prostate Weights in Lobund Wistar Rats Exposed to the Chemoprevention Protocol*

Diet	Body Weights (G)	Testes (G)	Dorsolateral Prostate (mg)
Zero Genistein in AIN-76A	362 ± 8	1.22 ± 0.15	319 ± 17
25 mg Genistein/kg AIN-76A	357 ± 4	1.24 ± 0.18	264 ± 17
250 mg Genistein/kg AIN-76A	369 ± 3	1.59 ± 0.20	242 ± 22

*Rats were exposed to genistein via the diet starting at conception. From day 50-66 postpartum, male offspring were gavaged with 33 mg Flutamide/kg BW. On days 67, 68, and 69, they were injected with 25 mg testosterone/kg BW. On day 70, all rats were injected with 42 mg NMU/kg BW into the dorsolateral prostate. One week after NMU administration, silastic implants of 25 mg of testosterone were started. Animals were killed when 117 days old.

Table 4. Prostate cancer incidence in Lobund-Wistar rats fed genistein in the diet

Treatment	Percent of Rats with Prostate Tumors	Tumor Invasive Adenocarcinomas	Intraductal Carcinomas
0 Genistein in AIN-76A diet	19/22 (86.4%)	17/22 (77.3%)	11/22 (55.0%)
25 mg Genistein/kg AIN-76A Diet	14/18 (77.8%)	11/18 (61.1%)	9/18 (52.9%)
250 mg Genistein/kg AIN-76A Diet	17/27 (63.0%)	12/27 (44.4%)[a]	8/27 (34.5%)

Lobund-Wistar rats were provided 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception. Male offspring were injected s.c. with 33 Flutamide/kg BW on days 50-66, with 25 mg testosterone/kg on days 67-69, with 42 mg NMU/kg into the dorsolateral prostate on day 70, and given testosterone implants of 25 mg each starting at day 77 (and replaced every 12 weeks). Animals were necropsied when 48 weeks old or when moribund. [a] $P < 0.05$ compared to 0 genistein in AIN-76A diet group (Fisher Exact Test).

Table 5. Genistein Concentrations in Blood and Prostates of Lobund-Wistar Rats Exposed to Genistein in the Diet From Conception until 70 Days Postpartum*

	Genistein Concentration			
	Blood (pmoles/ml)		Prostate (pmoles/g)	
Diet	Free	Total	Free	Total
Zero Genistein in AIN-76A	ND	9 ± 1	ND	85 ± 16
25 mg Genistein/kg AIN-76A	9 ± 1	60 ± 6	ND	230 ± 107
250 mg Genistein/kg AIN-76A	67 ± 7	861 ± 104	774 ± 273	775 ± 246

*Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum. Serum and dorsolateral prostate samples were processed and assayed for genistein concentrations by HPLC-MS. ND: not determined. Each value represents 6 samples/group.

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Dietary genistein suppresses chemically-induced prostate cancer development in Lobund-Wistar rats. Wang, J., Eltoum, I.-E. and Lamartiniere, C. A. *Departments of Pharmacology and Toxicology, and Pathology; Comprehensive Cancer Center. University of Alabama at Birmingham, Birmingham AL 35294.*

Asian men consuming a traditional diet high in soy products have a lower incidence of clinically manifested prostate cancer than Western men. We have investigated one of the components of soy, genistein, to protect against prostate cancer by feeding Lobund-Wistar rats 0, 25 and 250 mg genistein/kg AIN-76A diet, starting at conception. From day 50-66 postpartum, the male offspring were gavaged with 33 mg Flutamide/kg BW to effect chemical castration. On days 67, 68, and 69, they were injected with 25 mg testosterone/kg BW to stimulate mitosis. On day 70, all rats were anesthetized and 42 mg N-methlynitrosourea (MNU)/kg BW was injected into the dorsal prostate for cancer initiation. One week after MNU administration, silastic implants of 25 mg of testosterone were implanted (and replaced every 12 weeks) to stimulate mitosis and promote tumor growth. By 40 weeks of age, palpable prostate tumors were detectable. Animals were necropsied when 48 weeks old or when animals became moribund. The percent of prostate tumors that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250 mg genistein/kg diet were 77.3%, 61.1%, and 44.4%, respectively. Genistein did not alter body, prostate or testes weights or feed consumption. Male rats fed 0, 25 and 250 mg genistein/kg diet had serum genistein concentrations of 9, 60 and 861 pmol/ml, and prostate genistein concentrations of 85, 230 and 775 pmol/g tissue. We conclude that a diet yielding blood genistein concentrations in rats similar to those found in the blood of Asians eating a traditional soy-containing diet can protect against chemically-induced prostate cancer.

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Dietary Genistein Suppresses Chemically-induced Prostate Cancer in Lobund-Wistar Rats

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Abstract

Epidemiological reports suggest that Asians consuming a diet high in soy have a low incidence of clinically manifested prostate cancer. We have tested the hypothesis that life-time exposure to genistein, the primary isoflavone component of soy, is responsible for this protective effect. Lobund-Wistar rats were exposed to 0, 25 and 250 mg genistein/kg AIN-76A diet, starting at conception and continued until necropsy at 11 months. Male offspring were injected s.c. with Flutamide on days 50-66 and with testosterone on days 67-69, injected with N-methylnitrosurea into the dorsal prostate on day 70, and given testosterone implants, starting at day 77. Genistein in the diet inhibited the development of NMU-induced prostate intraductal carcinomas/invasive adenocarcinomas, in a dose dependent manner. Genistein did not alter body, prostate and testes weights. Male rats fed 0, 25 and 250 mg genistein/kg diet had serum genistein concentrations of 9, 60 and 861 pmol/ml, and prostate genistein concentrations of 85, 230 and 775 pmol/g tissue. We conclude that lifetime dietary genistein protected against chemically-induced prostate cancer development in rats.

Keywords: Genistein; Chemoprevention; Rat; Prostate; Cancer

1. Introduction

Asian men consuming a traditional diet high in soy products have a lower incidence of clinically manifested prostate cancer compared to American and European men [1-4]. Yet, Asians who emigrate to the United States and adopt a western diet lose this protection [5-8]. While the role of fat intake [9,10] and genetics [11] for cancer susceptibility are unclear, other nutritional components may play a significant role. Soy based diets are high in phytochemicals and quantitative results indicate that isoflavonic phytoestrogens are normal constituents of human urine from subjects consuming large amounts of soy products (tofu, soy flour, soy milk, tempeh, etc) [3,12]. The predominant phytoestrogen component of soy is genistein.

In vitro, genistein has been shown to have weak estrogenic properties [13-15], to be an anti-oxidant [16-18], to inhibit topoisomerase II [19], tyrosine kinases [20] and angiogenesis [21], and to induce cell differentiation [22,23]. Genistein has been reported to inhibit the growth of human and rat prostate cell lines [24]. Genistein also inhibits EGF-stimulated growth of LNCaP and DU-145 cells, and EGFR tyrosine autophosphorylation in DU-145 cells but not in LNCaP cells [25]. In general, genistein concentrations necessary to show these *in vitro* effects were 10-100 μM, concentrations which are 1-2 orders of magnitude higher than the circulating genistein levels in humans [26]. Hence, the relevance of these effects in *in vivo* remain to be determined.

Soy phytochemicals have been shown to inhibit growth of prostate cancer tumors in rodents injected with prostate cancer cells [27-29]. Pollard and Luckert [30] have shown that in Lobund-Wistar rats fed high-isoflavone-supplemented soy diet, the incidence of N-methylnitrosurea (NMU) induced prostate-related cancer was reduced and the disease-free period was prolonged by 27% compared with rats fed the same diet, but low in isoflavones.

The most susceptible rat model for prostate cancer is the Lobund-Wistar rat which spontaneously

develops a relatively high incidence (26% in an average of 26.6 months) of metastatic adenocarcinomas of the prostate complex and seminal vesicle [31]. In order to shorten the latency period and increase the incidence of tumors, researchers have used chemical carcinogens to induce tumorigenesis [30, 32-34]. Prostate tumor incidence has been increased and the latency shortened in Lobund-Wistar rats injected i.v. with NMU followed by a series of s.c. implants of testosterone propionate [34]. These tumors were induced in 50-90% of Lobund-Wistar rats with an average latency of 11.5 months. The histopathology of these tumors were similar to those that develop spontaneously, i.e. moderately differentiated metastasizing adenocarcinomas to the prostate complex and seminal vesicles. However the origin of these tumors was not localized to the dorsolateral prostate in the rat which is the homologue to the human prostate [35]. Recently, Schleicher et al. injected NMU orthotopically into the dorsolateral lobe of Wistar rats [36]. This resulted in 40% incidence of tumors.

We have demonstrated that genistein inhibits expression of the EGF-receptor in prostates of Lobund-Wistar rats [37]. Later, we reported that pharmacologic doses of genistein injected into Lobund-Wistar rats inhibited the growth and metastasis of a transplantable rat prostate carcinomas [27]. The goal of this investigation was to determine if lifetime exposure to genistein via the diet, including in *in utero*, could protect against chemically-induced prostate cancer initiated in the dorsolateral prostate.

2. Materials and methods

Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the UAB Animal Care Committee. Seven week old virgin female Lobund-Wistar rats were purchased from the National Cancer Institute. Animals were kept in a climate-controlled room with a 12 h light/12 h dark cycle. These animals were fed AIN-76A diet (Harlan Texlad, Madison WS) ± genistein and acclimated for two weeks in our facilities prior to mating. One female per proven Lobund-

Wistar stud, was co-habitated for 2 weeks. Chemically-synthesized genistein (98.5% pure by HPLC analysis) was generously provided to us by Roche, Basel, Switzerland. The AIN-76A diet is a semi-purified diet that does not contain soy. Furthermore, each batch of AIN-76A diet was analyzed by HPLC for isoflavones. The limit of detection for the isoflavones using HPLC was 10 pmol/g. Genistein was mixed in the diet at 25 mg/kg and 250 mg/kg AIN-76A diet by the laboratory of Dr. Clinton Grubbs (Nutrition Sciences, UAB). Previously, we showed that these dietary genistein concentrations resulted in serum genistein concentrations in rats similar to those of humans eating a traditional Asian diet high in soy (26, 38), and protected against chemically-induced mammary cancer in rats [38].

Group 1) Life-time AIN-76A diet (no genistein)

Group 2) Life-time exposure to 25 mg genistein/kg AIN-76A diet

Group 3) Life-time exposure to 250 mg genistein/kg AIN-76A diet

The protocol for the induction of prostate tumors in rats is a modification of that described by Schleicher et al [27,36]. From day 50-66 postpartum, one male offspring from each litter was gavaged with 33 mg Flutamide/kg BW (gift from Dr. Rudolph Neri, Scherring-Plough Research Institute, Kenilworth, NJ) in sesame oil (Sigma Chemical Co, St Louis, MO) to effect chemical castration. On days 67-69, the rats were injected with 25 mg testosterone/kg BW (Sigma Chemical Co.) to stimulate mitosis. On day 70, all rats were anesthetized with Ketamine/Xylazine, a midline abdominal incision was made, and 42 mg N-methylnitrosourea (NMU)/kg BW was administered via 2 injections into the dorsolateral prostate for cancer initiation. The NMU was dissolved in dimethylsulfoxide (250 mg/ml) and a beveled 25 gauge, 5/8 inch needle (Becton Dickson, Franklin Lakes, NJ) onto a 25 ul Hamilton syringe (Hamilton Co., Reno, NV) was used. One week after NMU administration, rats were given silastic implants (1.8 cm long x 0.078 inch inner diameter x 0.024 inch wall) (SF Medical, Hudson, MA), plugged with medical grade adhesive (Dow Corning, Midland MI) packed with 25 mg testosterone. The silastic implants were soaked for 2-4

hr and then overnight in PBS containing 1% BSA. The testosterone capsules were implanted s.c. in the scapular region using Ketamine/Xylazine anesthesia for the purpose of cancer promotion.

Animals were sacrificed and a detailed necropsy was carried out when they were 11 months old. Particular attention was paid to male genital and pelvic organs. Tumor size, origin and spread were noted. The prostate, seminal vesicles and metastatic lesions were histologically examined on routine hematoxylin-and-eosin stained sections. We staged the tumors into three classes: Stage I where the tumor was confined to the site of injection in the dorsolateral prostate, Stage II where the tumor involved any of the following adjacent organs: coagulating gland, seminal vesicles, rest of the prostate or bladder; and Stage III where the tumor extended beyond these organs. For tumor grading, we used Gleason's grading system, which is the standard method of grading prostate carcinoma in human and solely depends on architectural arrangement of tumor glands [39].

Concentrations of genistein and its metabolites from sera and prostate tissue were analyzed by HPLC-multiple reaction ion monitoring mass spectrometry [40]. The level of detection was 10 nM. Serum testosterone concentrations were determined using an in-house radioimmunoassay procedure [41]. Histomorphological evaluation of the tumors was carried out on coded slides by Dr. I.-E. Eltoum, a Board Certified Pathologist. Data were analyzed by One Way Analysis of Variance (ANOVA) using the Sigma Stat computer program (Jandel Scientific, San Rafael, CA).

3. Results

Rats fed the control diet, AIN-76A, and subjected to the carcinogenesis protocol developed 86.4% incidence of prostate tumors by 11 months of age (Table 1). Animals exposed to 25 and 250 mg genistein/kg diet had tumor incidence of 77.8%, and 63.0%, respectively. Except for two animals, one with ganglioneuroma and the other with malignant peripheral nerve sheath tumor, prostatic carcinoma was the

sole malignancy identified in all of these animals. The percent of tumors to the prostate that were classified as invasive adenocarcinomas in rats fed 0, 25 and 250 mg genistein/kg diet were 77.3%, 61.1%, and 44.4%, and the incidence of intraductal carcinomas were 55.5%, 52.9% and 34.5%, respectively (Table 1). Comparison of tumor invasive adenocarcinomas from rats exposed to 250 mg genistein/kg diet to no genistein in the diet was statistically significant ($P < 0.05$). In the majority of animals (55%), the tumors presented as Stage I (confined to the dorsolateral prostate), and 33% as stage II (tumor involved the seminal vesicle, or other prostates), and in few (12%) as stage III (distal metastasis). Genistein treatment had no effect on the stage of the tumors. Unlike human prostatic carcinomas, which show all Gleason's grades, prostatic tumors in these animals presented mostly as poorly differentiated carcinomas. They were composed of small irregular glands and cluster of atypical cells infiltrating a dense stroma. There was extensive acute inflammation, necrosis and calcification. Mucous secretions as well as signet cells were occasionally present. The histology did not vary between animals, or between stages. The lowest Gleason score was 6, and the majority between 8 and 10. We found no difference in Gleason's score of tumors in genistein-treated and -untreated animals. Mild to moderate dysplasia (prostatic intra-epithelial neoplasia) was seen less frequently in genistein-treated (63%) than -untreated (81%) animals. Prostate tumor weights from the 3 groups were not significantly different from one another (5.2 ± 1.8 grams). In animals with small tumors, the tumors were confined to the site of NMU injection, the dorsolateral prostate.

Frequently, the prostate tumors did involve the seminal vesicles. Occasionally, there was metastasis to the ventral prostate, the coagulating gland, the terminal portion of the vas deferens, lymph nodes, bladder, adrenal gland, diaphragm, kidney, liver, lungs, mesentery, and spleen. There was no evidence of metastasis to the bulbourethral gland, epididymis, testes, penis, bone or brain.

Exposure to genistein in the diet from conception until time of carcinogen administration at 70

days postconception did not cause significant alterations to body weights (Table 2). Likewise, perinatal exposure to genistein in the diet did not cause significant alterations to the testes and prostate weights. Continuation of genistein in the diet to adult rats (11 months old) on the carcinogenesis protocol did not significantly alter body, testes and dorsolateral weights compared to rats fed AIN-76A diet only (data not presented).

The daily feed consumption was measured in young male rats. All animals ate approximately the same amount of feed and gained the same amount of weight (Table 3). From this data we calculated that young male rats fed the low and high genistein containing diets ingested 1.80 mg and 19.25 mg genistein/day/kg BW, respectively.

Total (aglycone and conjugated) and free (aglycone) genistein concentrations were measured in the blood and prostates of rats exposed to dietary genistein from conception until 70 days old. An increase in dietary genistein content resulted in increased serum and prostate genistein concentrations (Table 4). In the sera of rats fed 25 mg and 250 mg genistein/kg diets, the free genistein concentrations were 15% and 8% of the total genistein, respectively. In the prostates of rats fed the high genistein-containing diet, the free genistein content was similar to the total genistein concentration. Free genistein was not measured in prostates of rats fed the low genistein containing diet. We conclude that genistein is readily bioavailable to the prostate as the aglycone.

4. Discussion

The following report takes into consideration rat models of prostate cancer, especially the one published by Schleicher et al. [36], and our previous genistein studies in Lobund-Wistar rats [27,37]. We chose Lobund-Wistar rats because they are most susceptible strain (highest incidence of spontaneously developing tumors: 26%). We initiated the carcinogenesis protocol relatively early in life (at 50 days for

the Flutamide and 70 days for the NMU) because prostate cancer in humans is initiated early, even though the long latency period results in this being a disease mainly of aging men. We used 33 mg Flutamide/kg BW as opposed to 50 mg/kg BW [36] because these were young rats for which we did not want significant toxicity during development. The Flutamide-induced chemical castration was followed by injections of testosterone to stimulate mitosis in the prostate. The NMU was injected into the dorsolateral prostate to enhance tissue specific carcinogenesis. This is consistent with preliminary experiments that demonstrated dye injected into the dorsolateral prostate localized to the dorsolateral lobes and did not leak out. The use of 25 mg testosterone instead of 100 mg testosterone [36], replaced every 12 weeks, to promote carcinogenesis was because we were concerned about the phytoestrogen, genistein, being able to counter the continuous influence of supraphysiological androgen concentrations.

Lobund-Wistar rats fed AIN-76A diet and treated with this carcinogenesis protocol developed 86% incidence of prostate tumors within 9 months of carcinogen exposure. Therefore, adding testosterone promotion to the intraprostatic injection of NMU doubled the incidence of prostate tumors [36]. The histopathological report revealed that 77% of these animals had invasive adenocarcinomas; 55% had intraductal carcinomas. In animals developing small tumors, the lesions were associated with the site of injection, the dorsolateral prostate. This confirms the specificity of cancer origin in this model. The prostate tumors did metastasize to other organs of the reproductive tract, i.e., ventral prostate, seminal vesicles, vas deferens, bulbourethral gland, coagulating gland. There was also metastasis to the bladder, adrenal gland, diaphragm, kidney, liver, lungs, lymph nodes, mesentery, and spleen. There was no evidence of metastasis to the bulbourethral gland, epididymis, testes, penis, bone or brain.

The doses of genistein used in this chemoprevention study were selected on the basis of blood genistein concentrations in rats compared to those measured in humans. Rats fed 25 mg and 250 mg genistein/kg AIN-76A diet were determined to have circulating total genistein concentrations of 60 and 861

pmol/ml, respectively. Asian men eating a traditional diet high in soy have been reported to have total genistein concentration of 276 pmol/ml in the blood [26]. Hence, these dietary concentrations resulted in serum genistein concentrations that "frame" the human circulating genistein concentration. Feed consumption studies showed that the 25 mg and 250 mg genistein/kg diet did not alter the amount of feed eaten by these rats. We have previously investigated the potential of perinatal genistein exposure to alter fertility and to cause toxicity to the reproductive tract in the female rat and have found no significant effect [38]. Likewise, in this study, we did not record any significant alterations to body, testes and prostate weights. In a separate study, no histomorphological alterations were observed in the male reproductive tracts of Sprague Dawley rats exposed to these dietary concentrations of genistein from conception until 70 days old [42]. Furthermore, Flynn et al. have reported that dietary genistein at 25 mg and 250 mg/kg AIN-76A diet fed to pregnant rats, beginning on gestational day 7 and the offspring continued until postnatal day 77, did not significantly alter gestational duration, total offspring/litter, total sex ratio, live pups/litter, live sex ratio and average weight/live pup [43] and nursing behavior [44]. We conclude that perinatal exposure to dietary genistein does not cause significant toxicity to male rat offspring.

Enzymatic hydrolysis of the serum with beta-glucuronidase and sulfatase, and HPLC-mass spectrometry analysis showed that only 15% and 8% of the genistein was free genistein (aglycone) from blood of rats fed 25 mg and 250 mg genistein/kg diet, respectively, demonstrating that most of the circulating genistein is conjugated. In the prostate of rats fed the high genistein containing diet (250 mg/kg diet), genistein concentrations were measured to be 775 pmole/g tissue. This is similar to the corresponding blood levels, 861 pmoles/ml. This shows that there is no genistein transport barrier to the prostate. Another significant finding is that all of the genistein in the prostate was in the free form (774 pmol/g), none was conjugated. This suggests that all of the genistein is available to the prostate for direct

biological action.

The fact that "high physiological" concentrations of genistein in the diet (250 mg genistein/kg) did not alter body, testes and prostate weights, but did provide protection against chemically-induced prostate cancer suggests that the primary action of genistein is not restricted to classical estrogen signaling. Administration of estrogen to male rats reduces the size of the accessory sex glands and circulating testosterone concentrations. We have previously reported that 3 weeks of genistein in the diet resulted in slight, but not significantly increased blood levels of testosterone [37]. This contrast is also seen between estrone's action to promote prostate cancer in the Lobund-Wistar rats [27], while genistein suppresses prostate cancer. We have previously reported that genistein in the diet inhibits expression of the EGF-receptor mass in prostates of Lobund-Wistar rats, but not its phosphorylation [37]. Inhibiting this pathway associated with cell proliferation may be a mechanism by which genistein (and soy) could suppress prostate cancer. Experiments into sex steroid dependent and independent signaling pathways, and down-stream events are necessary. Likewise, investigations into genistein timing of exposure (prenatal, neonatal, prepubertal and adult) are also warranted.

In summary, lifetime dietary genistein protected against chemically-induced prostate cancer development in rats. Dietary genistein resulted in serum genistein concentrations similar to blood genistein concentrations in Asian men eating a traditional diet high in soy [26]. Genistein was bioavailable to the prostate. Perinatal exposure to genistein in the diet did not cause significant toxicity to the offspring.

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Table 1

Prostate cancer incidence in Lobund-Wistar rats fed genistein in the diet

Treatment	Percent of Rats with Prostate Tumors	Intraductal Carcinomas	Tumor Invasive Adenocarcinomas
0 Genistein in AIN-76A diet	19/22 (86.4%)	11/22 (55.0%)	17/22 (77.3%)
25 mg Genistein/kg AIN-76A Diet	14/18 (77.8%)	9/18 (52.9%)	11/18 (61.1%)
250 mg Genistein/kg AIN-76A Diet	17/27 (63.0%)	8/27 (34.5%)	12/27 (44.4%)[a]

Lobund-Wistar rats were provided 0, 25 and 250 mg genistein/kg AIN-76A diet starting at conception. Male offspring were injected s.c. with 33 Flutamide/kg BW on days 50-66, with 25 mg testosterone/kg on days 67-69, with 42 mg NMU/kg into the dorsolateral prostate on day 70, and given testosterone implants of 25 mg each starting at day 77 (and replaced every 12 weeks). Animals were necropsied when 48 weeks old or when moribund. [a] $P < 0.05$ compared to 0 genistein in AIN-76A diet group (Fisher Exact Test).

Table 2

Body, testes and dorsolateral prostate weights in Lobund-Wistar rats exposed to genistein in the diet from conception until 70 days old

Diet	Body (G)	Testes (G)	Dorsolateral Prostate (mg)
Zero Genistein in AIN-76A	244 ± 8	2.30 ± 0.04	190 ± 4
25 mg Genistein/kg AIN-76A	256 ± 4	2.35 ± 0.04	188 ± 5
250 mg Genistein/kg AIN-76A	254 ± 3	2.33 ± 0.02	186 ± 5

Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum. These animals were not exposed to the chemopreventive protocol. Each value represents mean ± SEM of 8 samples/group.

Table 3

Dietary genistein consumption in Lobund-Wistar male rats

Diet	Average BW over Period	Grams of Diet Eaten/Day/Rat	Grams of Diet /Day/kg BW	Milligrams of Genistein Eaten/Day/kg BW
Zero genistein	232 ± 6	17.9 ± 0.7	77	0
25 mg genistein/kg	235 ± 6	17.0 ± 0.6	72	1.80
250 mg genistein/kg	231 ± 6	17.7 ± 0.8	77	19.25

Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum. Feed consumption was measured from 60-70 days postpartum. Each value represents mean ± SEM of 8 samples/group.

Table 4

Genistein concentrations in blood and prostates of Lobund-Wistar rats exposed to genistein in the diet

	Genistein Concentration			
	Blood (pmoles/ml)		Prostate (pmoles/g)	
Diet	Free	Total	Free	Total
Zero Genistein in AIN-76A	ND	9 ± 1	ND	85 ± 16
25 mg Genistein/kg AIN-76A	9 ± 1	60 ± 6	ND	230 ± 107
250 mg Genistein/kg AIN-76A	67 ± 7	861 ± 104	774 ± 273	775 ± 246

Dams were fed AIN-76A diet supplemented with 0, 25 and 250 mg genistein/kg diet and the offspring were continued to be fed this diet after weaning at day 21 through day 70 postpartum. These animals were not exposed to the chemopreventive protocol. Serum and dorsolateral prostate samples were processed and assayed for genistein concentrations by HPLC-MS. ND: not determined. Each value represents mean ± SEM of 6 samples/group.